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Contributions of the hippocampus and entorhinal cortex to rapid visuomotor learning in rhesus monkeys

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Abstract

The hippocampus and adjacent structures in the medial temporal lobe are essential for establishing new associative memories. Despite this knowledge, it is not known whether the hippocampus proper is essential for establishing such memories, nor is it known whether adjacent regions like the entorhinal cortex might contribute. To test the contributions of these regions to the formation of new associative memories, we trained rhesus monkeys to rapidly acquire arbitrary visuomotor associations, i.e., associations between visual stimuli and spatially directed actions. We then assessed the effects of reversible inactivations of either the hippocampus (Experiment 1) or entorhinal cortex (Experiment 2) on the within-session rate of learning. For comparison we also evaluated the effects of the inactivations on performance of problems of the same type that had been well learned prior to any inactivations. We found that inactivation of the entorhinal cortex but not hippocampus produced impairments in acquiring novel arbitrary associations. The impairment did not extend to the familiar, previously established associations. These data indicate that the entorhinal cortex is causally involved in establishing new associations, as opposed to retrieving previously learned associations.

Keywords

associative learning; fornix transection; temporary inactivation; medial temporal lobe

The hippocampal system — consisting of CA1-CA4, the dentate gyrus, the subicular complex and the fibers of the fornix and fimbria — is essential for certain types of associative learning, including arbitrary associations of a visual stimulus with a spatially directed response, as assessed by visuomotor learning (VML). Monkeys with either aspiration lesions of the hippocampus plus underlying cortex (Murray and Wise, 1996) or fornix transection (Rupniak and Gaffan, 1987; Brasted et al., 2003; Brasted et al., 2005) are impaired in acquiring such arbitrary associations. Findings from neurophysiological studies are consistent with the idea that the hippocampus, in particular the CA fields, are responsible for the acquisition of arbitrary stimulus association learning. Neurons in the hippocampus of monkeys show learning-related changes in activity during VML (Wirth et al., 2003) and

functional imaging studies in humans show BOLD responses in both hippocampus and entorhinal cortex correlated with the strength of associative learning of VML (Hargreaves et al., 2012).

Despite the foregoing, it is still not clear if — within the hippocampal system — it is the hippocampus that is necessary for associative learning in the VML task. Earlier studies that have specifically examined hippocampal contributions produced negative results. Brasted et al. (2005) trained monkeys on a version of VML with nonspatial responses and found that the rate of learning was significantly slowed by fornix transection. When aspiration removal of the entire hippocampal formation plus underlying parahippocampal cortex was added to fornix transection, however, there was no added impairment. In addition, monkeys with excitotoxic lesions of the hippocampus were not impaired on VML, despite the same group being impaired in another domain (Chudasama et al., 2008). Thus, severing the fibers of the fornix eliminates the entire contribution of the hippocampal system to this type of associative learning. Of course, many fibers in the fornix are directed neither into nor out of the hippocampus (Saunders and Aggleton, 2007). The essential neural substrate of VML could therefore lie outside the hippocampus, but contribute fibers to the fornix (e.g., entorhinal cortex).

Given the importance of understanding the contribution of the hippocampus and related structures to associative learning, we revisited the question of whether the hippocampus is required for rapid associative learning, using VML. To improve our chances of obtaining a definitive result, the present study had three key features. First, because a version of VML with spatial, as opposed to nonspatial, responses might arguably be more sensitive to hippocampal damage, we chose to employ VML with spatially directed responses. Second, because acute manipulations sometimes yield greater behavioral effects than permanent lesions, we assessed the effect on VML of temporary inactivations of the hippocampus. Third, given the evidence that entorhinal cortex contributes fibers to the fornix (Saunders and Aggleton, 2007), we planned to test the contribution to VML of the entorhinal cortex. Accordingly, we evaluated the effects of pharmacologically-induced reversible inactivations of either the hippocampus or the entorhinal cortex, bilaterally, on within-session learning of novel arbitrary associations and performance of well-learned associations of the same type.

Materials and Methods

Subjects

Four male rhesus monkeys (*Macaca mulatta*) weighing between 8.0 and 10.4 kg were studied. They were housed individually in cages and fed a controlled diet of monkey chow (Purina) supplemented with fruit. Water was always available. All procedures conformed to the Guide for the Care and Use of Laboratory Animals (1996, ISBN 0-309-05377-3) and were approved by the National Institute of Mental Health Animal Care and Use Committee. Of the four monkeys, two of them (monkeys B and I) participated both experiments. Monkey E only participated in Experiment 1, and monkey O only in Experiment 2.

Materials and Apparatus

During behavioral testing, monkeys sat in a primate chair inside a ventilated 3 ft × 2 ft × 4 ft test cubicle. The chair was held in place by clamps. A 15" touch-screen color monitor was affixed to the far wall of the box, 22 cm in front of the monkeys' eyes. Pellet dispensers (Med Associates) delivered rewards through a tube connected to a cup located centrally under the monitor, within the monkeys' reach. Touch-screen monitors and reward dispensers were under computer control. Rewards consisted of 190-mg banana-flavored pellets (TestDiet). White noise was played during testing to prevent distractions from extraneous sounds.

Stimuli used for shaping and instruction stimuli (IS) for the main task were chosen from a large collection of images of people, objects, and landscapes downloaded from the internet in the public domain and edited to 400 × 400 pixels. Images were stored as .bmp files and, when presented on the monitor, subtended around 40 degrees of visual angle. Target stimuli (TS) consisted of three identical, solid, white squares of dimensions 3 cm × 3 cm, which appeared in fixed locations on the monitor: 8 cm above, to the left, and to the right of the screen's midpoint.

Behavioral Design and Training

Shaping—After monkeys were accommodated to the primate chair, they were shaped to touch the monitor screen. Two automated programs were available for this purpose. In the first program, a single visual stimulus appeared in a central location on the monitor screen for 10 seconds, after which it disappeared and a reward was delivered. If the monkey touched the stimulus before the 10-second period ended, the trial terminated early and the monkey received a reward immediately. Stimuli appeared according to a 2-minute variable interval, and different stimuli appeared on each trial. There were 30 trials per session. Criterion was set at two touches on each of two consecutive sessions. In the second program, reward was made contingent on the animal's touching the stimulus on the screen. During each trial, the stimulus remained on the screen for a maximum of 30 seconds. If the monkey touched the stimulus on the screen, the trial was terminated, reward was delivered, and the intertrial interval (ITI) was initiated. If the monkey failed to touch the stimulus within 30 seconds, the stimulus disappeared, no reward was delivered, and the ITI was initiated. There were 50 trials per session, each separated by 30 seconds. Shaping was complete when the monkey touched the stimulus on the screen on at least 49 out of 50 trials for two consecutive days.

Once the monkeys had successfully learned to use the touch screen, they began training on VML.

Visuomotor Learning

The VML task required the monkeys to form arbitrary associations between visual stimuli and spatially directed motor responses. During each session, three distinct visual stimuli were mapped onto three different locations on the monitor, which served as response targets. Each trial began with the appearance of an IS on the monitor screen, which was selected randomly out of the three. The monkey was required to touch the IS, causing it to

immediately disappear. After 30 ms, the three identical TS appeared. The monkey was required to touch the TS location that corresponded to the IS. If the monkey chose the correct TS, the trial was terminated, reward was delivered, and an ITI of 3 sec was initiated. If the monkey chose one of the incorrect targets, the screen went blank, no reward was delivered, there was an ITI of 5 sec, and the next trial was a correction trial using the same IS. Correction trials continued until the monkey chose the correct target, at which point the monkey received a reward and moved on to the next trial. The longer ITI after an error, along with absence of food reward, served as penalties for incorrect responses.

Each session consisted of 50 trials (not including correction trials). In the final version of the task, we examined both the acquisition of problems using novel IS (Novel sessions) and performance of well learned problems using familiar IS (Familiar sessions). In Novel sessions monkeys were required to learn three new visuomotor mappings using three IS that they had never seen before. In Familiar sessions, monkeys had already completed a minimum of 60 sessions using the same IS-TS mappings, so they simply had to recall previously learned associations. In practice, however, monkeys always showed some relearning in the Familiar sessions, suggesting they tended to treat all stimuli as novel at the beginning of a session. Monkeys were trained at the rate of three sessions per day, with one Familiar session followed by two Novel sessions. There were four sets of Familiar IS-TS mappings; monkeys therefore encountered each set of Familiar mappings once or twice per week.

Surgical Procedures

After initial training, each monkey was implanted with a titanium headpost and stereotactically positioned magnetic resonance imaging (MRI)-compatible chamber (outer dimensions 30 × 50 mm). Aseptic procedures were used. Anesthesia was induced with ketamine hydrochloride (10 mg/kg, i.m.) and maintained with isoflurane (1.0-3.0%, to effect). Monkeys received isotonic fluids via an intravenous line and heart rate, respiration rate, blood pressure, expired CO₂ and body temperature were monitored throughout the procedure. Dexamethasone sodium phosphate (0.4 mg/kg, i.m.) was administered to reduce swelling and Cefazolin antibiotic (15 mg/kg, i.m.) was given to prevent infection. Both drugs were administered for one day before surgery as well as for one week after surgery. Monkeys received the analgesic ketoprofen (10-15 mg, i.m.) at the end of surgery and for two additional days. This was followed by 100 mg of ibuprofen for the next five days. Finally, in a separate surgical procedure, each monkey received a craniotomy above the infusion targets. Both the chamber placement and the location of the craniotomy were guided by structural MRI scans obtained prior to the surgery.

Infusion Procedures and Site Verification

Although muscimol is a standard agent for transient inactivation, its effects may persist for an additional 12 hours following administration. To eliminate carryover effects into the subsequent daily test session, we elected to use another GABA_A agonist, 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (THIP, Tocris/R&D Systems). The effects of both systemic (2.5 mg/kg, in canine model; Madsen et al., 1983) and intracerebral infusions (38 –

42 mM; Di Scala et al., 1983; Wilke et al., 2010) of THIP have been shown to persist for at least two hours after administration.

Accordingly, we temporarily inactivated either the hippocampus (Experiment 1) or the entorhinal cortex (Experiment 2), bilaterally, through intracerebral infusion of the GABA agonist THIP. The sites chosen for inactivation would be expected – on the basis of the topography of anatomical projections from perirhinal to entorhinal cortex and, in turn, from entorhinal cortex to the hippocampus – to receive object-based information from the perirhinal cortex (Fig 1). In the case of the hippocampus, the majority of projections from perirhinal cortex are indirect, via the entorhinal cortex (Witter et al., 1989; Suzuki and Amaral, 1994), although some are direct (Suzuki and Amaral, 1990).

Prior to the local infusions, a sterile field was prepared and the animal's head position was secured via the headpost. After the cap of the chamber was removed, a sterile rectangular grid ($46 \times 26 \times 10$ mm) with vertical channels at one mm intervals was inserted into the chamber. The channels in the grid allowed us to insert guide tubes and infusion cannulae to reliably target particular brain locations. For each site, we first inserted 24-gauge thin-wall stainless steel guide tubes with removable 30-gauge stylets. Once the guide tubes were in place, the stylets were removed and replaced with 30-gauge regular wall stainless steel cannulae (steel tubing, Component Supply Co.). The cannulae were fitted to Tygon microbore tubing connected to 10 μ l syringes (Hamilton). The drug or saline was pumped through the cannulae by Harvard Apparatus PHD 2000 infusion pumps. All materials (cannulae, guide tubes, plastic tubing, etc.) were sterilized prior to each use. Likewise, all compounds to be injected were dissolved in sterile 0.9% saline and filtered into sterile vials through 0.20 μ m sterile filters (Corning Inc.; final solution pH 7.0-7.5).

Infusions of THIP at a concentration of 18-38 mM were performed simultaneously at 2-3 sites per hemisphere in the hippocampus and 1 site per hemisphere in the entorhinal cortex. Although we initially used the lower dose (18 mM), which has been found to be behaviorally effective (Howland et al., 2011), we were motivated to use the higher concentration of THIP (38 mM) to rule out the possibility that the lack of effect following the hippocampal infusions was due to an ineffective dose. The effects did not differ across the doses tested so the data were pooled prior to analysis. THIP was infused at a rate of 0.25 μ l/minute for 6-11 minutes, followed by a 10-14 minute waiting period. For the majority of sessions with an experimental manipulation, we infused 1.5 μ l of the 18 mM concentration of the drug per site; this translates to 4.8 μ g THIP per site. Saline infusions with the same number of cannulae, volume, location, and timing served as a control. After the waiting period, the monkey immediately began behavioral testing, which lasted 15-60 minutes. Infusions were performed a minimum of seven days apart from one another, and THIP and saline infusion sessions were interleaved. A summary of the infusion sessions carried out in Experiments 1 (hippocampal inactivation) and 2 (entorhinal cortex inactivation) is provided in Tables 1 and 2, respectively.

Magnetic Resonance Scans

Prior to surgery, we obtained a T1-weighted (structural) MRI scan of each monkey's head for the purpose of guiding chamber placement. The monkeys were first given glycopyrrolate

(0.01 mg/kg, i.m.) to reduce secretions and then sedated with a mixture of ketamine (20 mg/kg, i.m.) and medetomidine (20 mcg/kg, i.m.). Monkeys were then placed in a nonferrous stereotaxic frame. Scans were acquired using a 3.0 T horizontal-bore scanner. The earbars of the stereotaxic frame, which were filled with Vitamin E, served as fiducial marks on the MR images.

After implantation of the headpost and chamber, each monkey received 2-4 additional T1-weighted scans in a 4.7 T vertical-bore scanner to permit accurate localization of infusion sites. The monkeys were sedated with a mixture of diazepam (0.5-15 mg/kg, i.m.) and ketamine (20 mg/kg, i.m.), and given glycopyrrolate (0.01 mg/kg, i.m.) to prevent secretions. Prior to scanning, the monkey was seated in a primate chair, the chamber lid was removed and the grid was inserted in the chamber. The chamber was then filled with the MR contrast agent gadolinium (0.5 M, Magnevist, Bayer HealthCare Pharmaceuticals Inc.) diluted in saline (1:1200-1:1500 v:v) and then resealed. There were two types of scans undertaken. The first scan was used to determine the appropriate location on the grid in the horizontal plane and the length of the cannula in the vertical plane required to reach our infusion sites. A second, similar structural MR scan was undertaken using the same procedures but this time it was preceded by infusion of gadolinium solution diluted in sterile saline (5 mM, see Asthagiri et al., 2011) into (or just above) the targeted brain areas. The infusion sites were thereby visible on the MR scan and could be confirmed before data collection began (see Fig. 1).

Data Analysis

Task performance was calculated as the percent of trials within a session that the monkey performed correctly on the first attempt (excluding correction trials). If a monkey did not complete all the trials in a session, all sessions from that day were discarded. We also assessed within-session learning. To do so, we divided each session into 10-trial blocks, and calculated a percent correct score for each individual block. The effects of inactivation on performance were determined by comparing scores obtained after infusion of THIP with those obtained after infusion of saline. Statistical analyses were performed using linear mixed effects models, with Subject (monkey) as a random effect. This was done to account for between-subject differences in baseline performance levels, and because this kind of model can accommodate an unbalanced experimental design. All statistical analyses were performed using the R statistical programming software.

To assess the effects of infusions on overall performance, the best-fitting model used Condition (Familiar, Novel), Infusion Type (THIP, Saline), and Condition by Infusion Type interaction as fixed effects, and Subject as a random effect. To examine within-session learning, we modeled the data from Familiar and Novel sessions separately. The best fitting models for both data sets used Infusion Type (THIP, Saline), Block (1-5), and Infusion Type by Block interaction as fixed effects, and Subject as a random effect.

Results

Task Acquisition and Performance

To assess learning in Familiar and Novel sessions, data were averaged across the 25 sessions of VML in both conditions (Familiar, Novel) administered immediately prior to each monkey's first infusion, and each session was divided into 5 blocks of 10 trials each. Figure 2 displays average learning curves by block.

Data were analyzed using a linear mixed effects model. The best-fitting model used Condition (Familiar, Novel), Block (1-5), and Condition by Block interaction as fixed effects, and Subject as a random effect. We found a significant effect of Condition [$F(1,987)=88.51$, $p<0.0001$], Block [$F(4,987)=61.6$, $p<0.0001$], and a significant interaction between Condition and Block [$F(4,987)=21.94$, $p<0.0001$]. Post-hoc tests revealed that performance on Familiar sessions was significantly better than performance on Novel sessions, but only during the first two blocks ($p<0.0001$), suggesting the animals were able to learn novel stimuli to a similar performance level as the familiar stimuli within a session. Performance during Block 1 was significantly worse than performance on all subsequent blocks ($p<0.0001$), an effect that was more pronounced in Novel sessions ($p<0.0001$) than Familiar sessions ($p=0.005$) but held for both (Tukey multiple comparisons of means, Bonferroni corrected).

Performance on the first trial of Novel sessions was not significantly better than chance [exact binomial $p(\text{one-tailed}) = 0.25$]. In contrast, performance on the first trial of Familiar sessions was significantly better than chance [exact binomial $p(\text{one-tailed}) < 0.0001$], reflecting previous learning of the visuomotor associations.

Thus, prior to receiving any infusions, monkeys were performing VML in a routine manner, with performance generally improving rapidly during the first 10 trials, and then stabilizing for the remainder of the session. In addition, as expected, they showed some benefit of repeated exposure to particular mappings, as performance on Familiar sessions was better overall, began above chance levels, and showed a weaker learning effect across blocks within session compared to Novel sessions.

Experiment 1: Effects of Hippocampal Inactivation on Visuomotor Learning

Hippocampal infusion sites are illustrated in Figure 1A and infusion sessions are summarized in Table 1. Average VML performance (measured by percent of trials correct on first try) for each condition is displayed in Figure 3 (insets). There was no effect of hippocampal inactivation on VML (effect of Infusion Type [$F(1,36)=0.68$, $p=0.42$]; interaction of Condition by Infusion Type [$F(1,36)=0.58$, $p=0.46$]). Similar to what we observed prior to any infusions, there was a significant effect of Condition [$F(1,36)=6.38$, $p<0.05$]; performance on Familiar sessions was significantly better than performance on Novel sessions ($p<0.01$, Tukey multiple comparisons of means, Bonferroni corrected).

We also examined within-session learning across blocks, i.e., the learning rate. Figure 3 displays average learning curves for VML sessions after hippocampal infusion of THIP and Saline. For Familiar sessions, there was a significant effect of Block [$F(4,48)=7.85$,

$p < 0.001$], with performance during Block 1 significantly worse than performance during subsequent blocks (Block 2, $p < 0.01$; Block 3, $p < 0.001$; Block 4, $p < 0.01$; Block 5, $p < 0.001$; Tukey multiple comparisons of means, Bonferroni corrected), but there was no effect of inactivation (interaction between Infusion Type and Block [$F(4,48)=0.76$, $p=0.56$]). Similarly, for Novel sessions, there was also a significant effect of Block [$F(4,138)=5.3$, $p < 0.001$]; performance during Block 1 was significantly worse than performance during Block 3 ($p < 0.01$), Block 4 ($p < 0.05$), and Block 5 ($p < 0.001$) (Tukey multiple comparisons of means, Bonferroni corrected). As was the case for Familiar sessions, there was no effect of inactivation (interaction between Infusion Type and Block [$F(4,138)=0.48$, $p=0.75$]).

Thus, for both Novel and Familiar VML sessions, hippocampal inactivation affected neither overall performance nor the rate of within-session learning.

Experiment 2: Effects of Entorhinal Cortex Inactivation on Visuomotor Learning

Entorhinal cortex infusion sites are illustrated in Figure 1B and infusion sessions are summarized in Table 2. Average performance (measured by percent of trials correct on first try) for each condition is displayed in Figure 4 (insets). In contrast to our findings with hippocampal inactivation, entorhinal cortex inactivation significantly impaired VML performance. Specifically, inactivation of entorhinal cortex impaired performance in Novel compared to Familiar sessions. Analyses of monkeys' behavior after THIP infusions revealed that performance after THIP infusions was significantly worse relative to performance after Saline infusions (effect of Infusion Type [$F(1,45)=24.75$, $p < 0.0001$], Tukey multiple comparisons of means, Bonferroni corrected). As before, performance on Familiar sessions was significantly better than performance on Novel sessions (effect of Condition [$F(1,45)=17.46$, $p < 0.001$], Tukey multiple comparisons of means, Bonferroni corrected). Despite there being a lack of an interaction between Condition and Infusion Type ($[F(1,45)=2.75$, $p=0.10]$), we conducted additional analyses to determine whether inactivation of entorhinal cortex had a greater effect in Familiar or Novel sessions. Post hoc tests showed that THIP infusions affected Novel sessions ($F(1,30)=39.65$, $p < 0.001$), but not Familiar sessions ($F(1,13)=1.53$, $p=0.24$). These data suggest that entorhinal cortex is causally involved in establishing new associations, as opposed to retrieving previously learned associations.

We also examined the effects of entorhinal cortex inactivation on within-session learning across blocks. Figure 4 displays average learning curves for VML sessions after entorhinal cortex infusion of THIP and Saline. For Familiar sessions, there was no effect of Block [$F(4,73)=0.84$, $p < 0.51$], and no effect of inactivation (interaction between Infusion Type and Block [$F(4,73)=0.71$, $p=0.59$]). For Novel sessions, however, there was a significant effect of Block [$F(4,158)=9.00$, $p < 0.0001$] and a significant effect of inactivation (interaction between Infusion Type and Block [$F(4,158)=2.85$, $p < 0.05$]). Post-hoc tests revealed that, as expected, during Saline sessions performance during Block 1 was significantly worse than during all subsequent blocks (Block 2, $p < 0.01$; Block 3, $p < 0.001$; Block 4, $p < 0.0001$; Block 5, $p < 0.001$; Tukey multiple comparisons of means, Bonferroni corrected). By contrast, during THIP sessions, there were no significant differences in performance across blocks,

suggesting a lack of within-session learning (Tukey multiple comparisons of means, $p > 0.1$, Bonferroni corrected).

Thus, inactivation of the entorhinal cortex impaired overall performance on Novel VML sessions, and reduced the within-session learning rate during Novel sessions.

Effects of hippocampal and entorhinal cortex inactivation on rules and strategies

Our prior studies indicated that monkeys employed behavior-guiding rules during VML. Specifically, there is evidence that monkeys employ *repeat-stay*, *lose-shift* and *change-shift* rules, application of which boosts performance above chance levels even in the absence of associative learning (Wise and Murray, 1999; Bussey et al., 2001). It is possible that even though temporary inactivation of the hippocampus did not cause any differences in overall performance or learning rate, it might have subtly altered monkeys' use of strategies. To assess possible contributions of the hippocampus and entorhinal cortex to implementation of behavior-guiding rules, we examined the use of these rules by monkeys in the present study. To ensure that learning would not contaminate our measure of rule application, we analyzed only trial 2 of Novel sessions.

Monkeys were found to use both the *repeat-stay* and the *lose-shift* rules. The *repeat-stay* rule entails making the same response (i.e., selecting the same TS) on the second trial as on the first if the same IS occurs. Here, the monkeys benefit by retaining the IS-TS information in short-term memory. The monkeys followed the previous choice 74% of time; this is substantially greater than the chance level of performance, which is 33% because the three IS were randomly selected for each trial. Conversely, there was a 67% chance that monkeys would encounter a different stimulus on the second trial than seen on the first. We called these “change” trials. The *change-shift* strategy relies on monkeys shifting their response (i.e., selecting a different TS) if the second trial is a change trial and if they performed correctly in the first trial. (We used a correction procedure, so the monkeys always terminated a trial with a correct response before proceeding to the second trial.) Finally, the *lose-shift* strategy relies on monkeys shifting their response (i.e., selecting a different TS) on a correction trial after an error, when their first attempt in the first trial was wrong. For change trials and correction trials after an error, a random strategy would yield 67% shift choices. Whereas monkeys employed the *lose-shift* strategy almost 100% of time, they did not use the *change-shift* strategy significantly more often than expected by chance with the exception of the condition of receiving saline infusions in the entorhinal cortex. There were no significant differences between the THIP and the Saline conditions for any strategies in either experiment (all $p > 0.05$).

Discussion

The hippocampal system has been shown to be essential for the acquisition of arbitrary associations. Here, to gain more precise information regarding the neural substrates of associative learning, we examined the role of the hippocampus and entorhinal cortex in VML. We found that inactivation of the entorhinal cortex disrupted acquisition of arbitrary visuomotor associations but left intact performance of already-learned associations. By contrast, inactivation of the hippocampus disrupted neither acquisition nor performance. An

important question for future research is whether either the hippocampus or entorhinal cortex contributes to other kinds of associative memory, such as the formation of visual stimulus-stimulus associations. Although lesions of the hippocampal formation have been reported to be without effect on visual-visual associative learning in monkeys (Murray et al., 1993), additional work is required to draw firm conclusions on this point (Wise and Murray, 1999); studies examining the effects of selective entorhinal cortex lesions on visual-visual learning have yet to be carried out.

The lack of effect of the hippocampal inactivation on visuomotor learning is consistent with the report of Brasted and colleagues (Brasted et al., 2005), who found that permanent excitotoxic lesions of the hippocampus failed to disrupt VML in the context of nonspatially directed responses. Thus, the present study replicates, using acute, reversible inactivations, the lack of effect of permanent excitotoxic hippocampal lesions. This is despite the fact that reversible manipulations can in many cases have a greater impact relative to static damage (Voytko, 1986; Horel et al., 1987), presumably because there is little or no possibility of functional compensation following acute manipulations. In addition, the present findings extend the negative outcome to a version of VML with spatially directed responses. These data therefore strongly suggest that the hippocampus proper is not necessary for the rapid acquisition of arbitrary associations between visual stimuli and spatially directed responses.

In considering the lack of effect of hippocampal inactivations, two additional issues are important to explore. First, could the lack of effect be due to an ineffective drug, or dose of drug? Because the same dose of THIP has been shown to be effective elsewhere in the brain (present study, Wilke et al., 2010), this account seems unlikely. In addition, a single hippocampal infusion per hemisphere of kynurenic acid, a glutamate receptor antagonist, has been reported to disrupt performance of macaques on another task (Forcelli et al., 2014), so the relatively small number of infusion targets per hemisphere (typically two) likewise seems unlikely to account for our failure to observe impairments. Second, could our infusions miss the key part of the hippocampus? Our infusion sites in the hippocampus were chosen to be in the anterior half. This is in keeping with several studies that have suggested the anterior part of the hippocampus is involved in associative learning and relational memory (Schacter and Wagner, 1999). In human functional imaging studies, the anterior hippocampus is active during encoding (Davachi and Wagner, 2002; Giovanello et al., 2004; Chua et al., 2007) and retrieval (Pihlajamäki et al., 2003) of associative information. More importantly, the hippocampal infusion sites overlapped with the recording locations of Wirth et al. (2003), who recorded learning-related activity in macaque hippocampus during VML. Finally, the hippocampal infusion sites were specifically chosen to be in areas that received visual sensory information about objects from perirhinal cortex, indirectly, via the entorhinal cortex (Witter et al., 1989; Suzuki and Amaral, 1994). Thus the lack of VML deficits from its inactivation seems unlikely to be due to poor placement of the infusions. Still, we cannot rule out the possibility that inactivations affecting the posterior hippocampus, or all of the hippocampus, would have produced an impairment.

Our inactivation of a limited region within entorhinal cortex produced significant learning deficits. Although the bulk of visual sensory inputs to entorhinal cortex are derived from perirhinal and parahippocampal cortex, and these sources of sensory input are typically

emphasized in primate studies, the entorhinal cortex has reciprocal anatomical connections with a wide variety of multimodal cortical fields (see Aggleton, 2012; Murray and Wise, 2012, for review). Given that fornix transection reliably produces impairments in VML (Rupniak and Gaffan, 1987; Brasted et al., 2003; Brasted et al., 2005), but hippocampal damage does not (Brasted et al., 2005; present study), it now seems likely that the entorhinal cortex projections through the fornix, whether direct or indirect via the subiculum, make an essential contribution to VML. The wider circuitry contributing to VML is, as yet, unidentified, but may include mammillary bodies, portions of the anterior thalamus, and portions of medial prefrontal cortex, all targets of projections arising from neurons in the entorhinal cortex or subicular complex (Aggleton et al., 1986; Aggleton et al., 2005; Munoz and Insausti, 2005). Not surprisingly, at least the mammillary bodies, anterior thalamus, and fornix are the same regions found to be essential for acquisition of the object-in-place scenes task (Parker and Gaffan, 1997a; Parker and Gaffan, 1997b), which shares many elements with VML (e.g., spatially directed movements, multiple problems being learned concurrently, etc.).

One drawback of the present study is the lack of a test for site specificity. In the present study, for example, one concern is whether our infusions into the entorhinal cortex might have had their positive effects due to drug leaking into the amygdala. This is possible because the amygdala is located immediately dorsal to the entorhinal cortex, and the infusion needle passes through this structure (see Fig. 1B). Although we did not directly investigate the effect of amygdala inactivations in the present study, we found in an earlier study that aspiration lesions of the amygdala had no effect on acquisition of visuomotor associations in a highly similar task (Murray and Wise, 1996). In addition, the MRI scans in which gadolinium-saline was infused into the entorhinal cortex to verify localization of the infusion sites (Fig 1B) also provide evidence that there was little reflux of the drug back up the infusion cannula. We therefore conclude that amygdala involvement in the present study was unlikely to be responsible for the impairment after THIP infusions targeting the entorhinal cortex.

Several recent findings have contributed to understanding the role of the entorhinal cortex in spatial processing. The discovery in rodents of grid cells (Hafting et al., 2005), border cells (Solstad et al., 2008), and head-direction cells (Sargolini et al., 2006) suggests that the entorhinal cortex can encode relative locations within a 2-dimensional foraging field. Grid cells have also been described in monkeys, where they appear to encode space during visual exploration, even without locomotion (Killian et al., 2012). Similar phenomena have been reported in the entorhinal cortex of humans (Doeller et al., 2010; Jacobs et al., 2010). These latter findings suggest that the entorhinal cortex might contribute to VML by representing the spatial layout of the visual field in which the monkey makes its reaching movement.

The functions of the primate entorhinal cortex extend beyond the spatial domain, however. We have shown, for example, that the entorhinal cortex is essential for the maintenance, in visual short-term memory, of objects (Meunier et al., 1993). For VML to occur, the monkey must maintain nonspatial information in short-term memory. The reason is that until feedback arrives in the form of reward or nonreward, there is no way to learn whether the choice of a given target was correct or incorrect. In the present experiment, the monkey

could see its hand at the TS when the feedback arrived, but the IS had disappeared prior to the onset of the target array. So an impairment in maintaining nonspatial information about the IS would be expected to severely disrupt learning. Importantly, retrieval of previously learned visuomotor associations would not be affected by this impairment because eventually the instruction–action–outcome associations become encoded in long-term memory. This idea is consistent with the findings that the entorhinal cortex is active in situations that require humans to retrieve recently learned object-in-place information by reaching to the correct location on a monitor screen (Owen et al., 1996) and that the entorhinal cortex contributes to the representation of conjunctions between particular objects, actions and outcomes (Hindy and Turk-Browne, 2013).

Another possibility is that inactivation of the entorhinal cortex produced a disruption of visual perceptual processing. Notably, the infusion sites in the entorhinal cortex are located several millimeters from the perirhinal cortex. Accordingly, it is extremely unlikely that our infusions involved perirhinal cortex directly. Although perirhinal cortex contributions to visual perceptual processing are well documented (Buckley et al., 2001; Bussey et al., 2002; Bussey et al., 2003), similar studies evaluating the contributions of entorhinal cortex have not been carried out. In the case of object recognition as measured by delayed matching- or nonmatching-to-sample, there is a mild and transient effect of entorhinal cortex lesions (Meunier et al., 1993; Leonard et al., 1995), suggesting a small contribution of entorhinal cortex to visual perceptual processing. In the present study, however, the fact that monkeys performed comparably on Familiar sessions after infusions of Saline and THIP into entorhinal cortex suggests that visual perception was not impaired. Future studies of entorhinal cortex function in monkeys should include a range of tasks to assess visual perceptual abilities directly, as has previously been done in investigations of perirhinal cortex (Baxter and Murray, 2001; Buckley et al., 2001; Bussey et al., 2002; Bussey et al., 2003) for object processing and hippocampus (Graham et al., 2010) for spatial processing.

Our prior studies also found that lesions of hippocampus plus underlying cortex left intact the ability to use behavior-guiding rules (Wise and Murray, 1999). By contrast, aspiration lesions of the ventral prefrontal plus orbital cortex severely disrupted use of preoperatively acquired rules (Bussey et al., 2001). In the present study, we found no evidence that infusions within either the hippocampus or the entorhinal areas had a significant impact on monkeys' rule applications. Thus, the pattern of impairment after entorhinal cortex infusions matches that seen after either fornix transection or aspiration lesions of the hippocampus plus underlying parahippocampal cortex.

Our results are especially surprising in view of the fact that the hippocampus has been so strongly identified with both spatial and nonspatial associative learning (Davachi, 2006; Konkel et al., 2008). A recent study of the neurophysiological correlates of VML in humans and monkeys (Hargreaves et al., 2012) showed that both the entorhinal cortex and hippocampus carry signals about relevant task events (e.g., stimulus novelty, trial outcome). These findings do not establish causal relationships, however, and the present results argue against a necessary role for the hippocampus in this form of learning. Notably, mirroring the findings from the present study, the analysis by Hargreaves and colleagues (2012) of local field potentials (LFPs) in monkeys performing VML revealed a relationship between LFPs

and learning strength for entorhinal cortex but not hippocampus. Together with anatomical findings (Aggleton, 2012; Murray and Wise, 2012), our results show that the entorhinal cortex does not function solely or even primarily as a ‘gateway’ to the hippocampus, but instead has its own specialized functions, to which the hippocampus does not contribute.

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Figure 1.

Localization of infusions in the hippocampus and entorhinal cortex. **A.** Left panel: Structural MR image in the sagittal plane showing region of the hippocampus targeted for infusions. Monkeys typically received 2 infusions in each hemisphere spaced 5 mm apart. Center and right panels: Structural MR images showing the site of infusion of an MR-contrast agent (mixture of gadolinium and saline) into the hippocampus in monkey B to verify target locations. The infusion site is manifest as a region of white hypersignal, denoted by the yellow arrows, in both coronal (center panel) and sagittal (right panel) views. **B.** Left panel: Structural MR image in the coronal plane showing region of the entorhinal cortex targeted for infusions. Monkeys typically received 1 infusion per hemisphere. Center and right panels: Structural MR images showing the site of infusion of an MR-contrast agent (mixture of gadolinium and saline) into the entorhinal cortex in monkey I to verify target locations. Conventions as in **A.**

Figure 2.

Mean percent error over the five 10-trial blocks of VML (N = 4). Curves show the within-session reduction in error (mean percent error $\pm$ SEM) for each condition (Familiar, Novel) averaged over the 25 sessions per condition that were administered immediately prior to infusions. Error rate was significantly higher for Novel sessions compared to Familiar sessions during Block 1 and Block 2 (both $p < 0.0001$), but not during Block 3, Block 4, or Block 5 (all $p > 0.41$). Inset shows mean percent error ($\pm$ SEM) for the same sessions collapsed across blocks.

Figure 3.

Mean percent error over the five 10-trial blocks of VML after bilateral infusion of THIP or Saline into the hippocampus ($N = 3$). Curves show the within-session reduction in error (mean percent error $\pm$ SEM) for each condition (Familiar, Novel) averaged over all hippocampal infusion sessions. Insets show mean percent correct ($\pm$ SEM) for the same sessions collapsed across blocks. There were no significant differences on performance after THIP vs. Saline infusion for either Familiar or Novel sessions.

Figure 4.

Mean percent error over the five 10-trial blocks of VML after bilateral infusion of THIP or Saline into entorhinal cortex ($N = 3$). Curves show the within-session reduction in error (mean percent error $\pm$ SEM) for each condition (Familiar, Novel) averaged over all infusion sessions. Performance after THIP infusion was significantly impaired relative to performance after Saline infusion during Block 2 ($p < 0.001$), Block 3 ($p < 0.05$), and Block 4 ($p < 0.001$) of Novel sessions. There were no other significant differences in performance between THIP and Saline for any of the blocks (Tukey multiple comparisons of means, Bonferroni corrected). Insets show mean percent correct ($\pm$ SEM) for the same sessions collapsed across blocks.

Table 1

Summary of infusion sessions in Experiment 1.

Infusion Type	THIP				Saline		
	Infusion Days	Familiar Sessions	Novel Sessions		Infusion Days	Familiar Sessions	Novel Sessions
Monkey							
Monkey E	5	3	12		1	1	2
Monkey B	2	2	4		1	1	2
Monkey I	4	4	8		1	1	2
TOTAL	11	9	24		3	3	6

Table 2

Summary of infusion sessions in Experiment 2.

Infusion Type	THIP				Saline			
	Infusion Days	Familiar Sessions	Novel Sessions		Infusion Days	Familiar Sessions	Novel Sessions	
Monkey								
Monkey O	4	4	8		2	2	4	
Monkey B	3	3	6		2	2	4	
Monkey I	4	4	8		2	2	4	
TOTAL	11	11	22		6	6	12	